Novel peptidoglycan-based diagnostic devices for detection of wound infection

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Abstract

Detection of wound infection is based on evaluation of the well-known signs of inflammation like *rubor* (redness), *calor* (heat), *tumor* (swelling), and *dolor* (pain) by medical doctors and/or time-consuming procedures requiring special machinery. There is currently no rapid diagnostic device available for the indication of wound infection, which would especially be helpful in home care of chronic ulcer patients. In this study, a new concept for a fast diagnostic tool for wound infection based on lysozyme and elastase triggered release of dye from a peptidoglycan matrix was investigated. The matrix consisted of alginate/agarose and peptidoglycan covalently labeled with Remazol brilliant blue. Lysozyme activity in postoperative wounds and decubitus wound fluids was significantly elevated upon infection (4830 ± 1848 U mL^{-1}) compared to noninfected wounds (376 ± 240 U mL^{-1}). Consequently, incubation of 8% (w/v) labeled agarose/peptidoglycan blend layers with infected wound fluid samples for 2 h at 37 °C resulted in a 4-fold higher amount of dye released than measured for noninfected wounds. For alginate/peptidoglycan beads, a 7-fold higher amount of dye was released in case of infected wound fluid samples compared to noninfected ones. Apart from lysozyme, proteases [i.e., gelatinase matrix metalloproteinase MMP-2 and MMP-9 and elastase] were detected in wound fluids (e.g., using Western blotting). When dosed in ratios typical for wounds, a slight synergistic effect was measured for peptidoglycan hydrolysis (i.e., dye release) between lysozyme and these proteases. Incubation of a double-layer system consisting of stained and nonstained peptidoglycan with infected wound fluids resulted in a color change from yellow to blue, thus allowing simple visual detection of wound infection.

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1. Introduction

Wound infection has been shown to impair wound contraction in both acute and chronic wounds (Hayward et al., 1992; Stenberg et al., 1991). However, currently, detection of wound infection is based on evaluation of the well-known signs of inflammation like *rubor* (redness), *calor* (heat), *tumor* (swelling), and *dolor* (pain) by medical doctors and/or time-consuming procedures requiring special machinery (Diegelmann and Evans, 2004). There is currently no rapid diagnostic tool available for the indication of wound infection, which would especially be helpful in home care of chronic ulcer patients. Chronic ulceration can affect any anatomical region—the most common site is the lower limb. The estimated prevalence of active leg ulceration in Europe is at least 0.1–0.3% (Callam et al., 1985; Nelzen et al., 1991). Wound healing involves a complex interaction between epidermal and dermal cells, the extracellular matrix, controlled angiogenesis, and plasma-derived proteins—all coordinated by an array of cytokines and growth factors.

This dynamic process is classically, but somewhat artificially, divided into 3 overlapping phases—inflammation, proliferation, and remodeling (Clark, 1996). Inflammation plays a pivotal role in all phases of the normal wound-healing response following the initial injury. The inflammatory response will act to recognize and eliminate potential pathogens and foreign material (Jones et al., 2004).

Infection has been defined as the product of the entrance, growth, metabolic activities, and resultant pathophysiologic effects of microorganisms in the patient's tissues (Robson, 1997). When this combination of events results in excessive amounts of bacteria in tissue, the balance or equilibrium is upset and infection ensues. High levels of bacteria not only result in infection but also have been demonstrated to impair every process of the wound healing scheme (Robson, 1997), (Stadelmann et al., 1998). Consequently, early detection of wound infection is essential in wound care.

Wound infection can be characterized by the identification of the causative organism(s) after wound swabbing (Collier, 2004). Serum investigations involve small amounts of blood being obtained from the patient to identify elevated white cell counts and elevated levels of serum C-reactive protein, a protein normally not found in the serum but present in many acute inflammatory conditions and with necrosis. However, both methods are not suitable for rapid diagnosis of a chronic wound infection (Krasner, 1990).

Furthermore, neutrophils are a predominant cell marker in the wound within 24 h after injury. The major function of the neutrophils is to remove foreign material, bacteria, and nonfunctional host cells and damaged matrix components that may be present in the wound site (Hart, 2002; Sylvia, 2003). Bacteria release chemical signals, attracting neutrophils, which ingest them by the process of phagocytosis (Tschaikowsky et al., 1993). Enzymes and other constituents of human neutrophil granules are, among others, myeloperoxidase, defensins, elastase, cathepsin G, phospholipase A_2, and lysozyme, which are partly released (Yager and Nwomeh,1999).

One of the biological functions of lysozyme is believed to be a self-defense against infection by bacteria (Osserman et al., 1973). Lysozyme (EC 3.2.1.17), known as muramidase or N-acetylmuramide glycanhydrolase, is an enzyme capable of hydrolyzing glycosidic linkages between the N-acetylmuramic acid and N-acetylglucosamine residues of the bacterial cell wall peptidoglycan (Salton, 1961). Many of the assays for the determination of lysozyme activity are based on the hydrolysis of the *Micrococcus lysodeikticus* cell wall and are thus a function of muramidase activity of the enzyme (Shugar, 1952). Besides the commonly used turbidimetric assay, *Micrococcus luteus* cells modified with Remazol brilliant blue R (RBB) were described by Ito et al. (1992) and Hardt et al. (2003). This method provides a more sensitive method applied for the determination of the concentration of lysozyme in serum (Ito et al., 1992), being accurate enough for screening many samples.

It is well known that any deviation of the lysozyme concentration from the normal level in serum or urine is related to diseases. For example, a high level of lysozyme was observed in the urine of patients with monocytic leukemia (Osserman and Lawlor, 1966). Serum levels may be raised in chronic granulomatous inflammatory disorders including tuberculosis and sarcoidosis (Pascual et al., 1973; Tomita et al., 1999). These observations indicate that the measurement of the concentration of lysozyme in serum or urine is useful for the diagnosis or screening of diseases. In addition, these clinical studies have suggested that increase of this enzyme is a serum marker of active chronic inflammation (Selroos and Klockars, 1977; Torsteinsdottir et al., 1999; Turton et al., 1979).

It is widely assumed that wound fluid has the potential to provide important biochemical information that can be used as a diagnostic indicator providing information for determining the overall status of a wound and for monitoring the progression of wound healing. Furthermore, wound fluid can be used to monitor specific parameters that have been identified as prognostic indicators or are targets of a particular therapy (Mani, 1999; Trengove et al., 2002; Yager et al., 2007).

Because there is a strong need for a fast prognostic aid that would assist in predicting clinical infection of a wound before obvious clinical symptoms, we have determined lysozyme levels in different types of chronic wounds. On the basis of these data, we have investigated different enzyme-responsive devices for simple detection of this enzyme and, as a consequence, the development of a rapid and simple diagnostic system for detection of wound infection.

2. Materials and methods

2.1. Sample collection and preparation

Wound fluid from 10 postoperative wounds, 8 decubitus ulcer wounds, and 8 blisters were collected and analyzed by biochemical techniques. Exudate samples were collected directly from exudating wound surface by using needles or a spoon before the daily treatment. Four samples were collected during vacuum-assisted closure therapy in special canisters without starch. The samples were centrifuged 3 times at $420 \times g$ for 20 min to remove cells and tissue material and stored at −20 °C until analysis. Thirteen out of these 26 wounds were described as clinically infected by the attending doctors. The patients studied so far were grouped and labeled with the following abbreviations (followed by a number): i = infected wound; n.i. = noninfected wound. Permission to collect wound fluid was obtained from the ethics committee of the Medical University of Graz.

2.2. Enzyme activities measured directly in wound fluid

2.2.1. Lysozyme activity

Lysozyme activity was directly determined in wound fluids using a modified method as described by Shugar (1952).

The assay is based on the determination of the lysis of *M. lysodeikticus* cells monitored turbidimetrically at 450 nm. *M. lysodeikticus* cells were suspended in 0.1 mol/L KH_2PO_4 buffer (pH 7.0) to obtain an approximate concentration of 0.05% (w/v). Ten microliters of sample or enzyme standard solution was added to 290 µL of this suspension to start the reaction. The initial rate of increase in transparency was monitored every 60 s at 450 nm and 25 °C.

2.2.2. Gelatinase activity

Gelatinase activity was measured with a commercial gelatinase kit (Enzcheck Gelatinase/Collagenase Assay Kit, Invitrogen, Carlsbad, CA, USA). As substrate gelatin, DQ Collagen Type IV Fluorescein Conjugate (Invitrogen, Carlsbad, CA, USA) was used. For measurement, 80 µL of reaction solution was mixed with 20 µL substrate solution and 100 µL diluted sample or collagenase from *Clostridium histolyticum*. The resulting fluorescence was measured at an excitation wavelength of 495 nm and at an emission wavelength of 515 nm, in black 96-well plates.

2.2.3. Elastase activity

Elastase activity is determined by measuring the cleavage of *N*-methoxysuccinyl-ala-ala-pro-val-*p*-nitroanilide as the chromogenic substrate as already described by Trengove et al. (1999). A solution of 1 mmol/L *N*-methoxy-succinyl-ala-ala-pro-val-*p*-nitroanilide (diluted in DMSO) in 0.1 mol/L HEPES buffer (pH 7.4, containing 0.5 mol/L NaCl) is used. To 5 µL sample (wound fluid) or commercial elastase (0.1–1 U/mL), 100 µL of the substrate solution is added. Substrate degradation is continuously monitored by measuring the increasing absorbance at 405 nm every 60 s at a temperature of 30 °C. The initial speed of the reaction (linear range) is used for calculations.

2.3. Zymography

Zymography was carried out according to an adapted and modified method of Ito et al. (1992) and Hardt et al. (2003). Polyacrylamide gel electrophoresis was performed using 10.0% (w/v) separating gels and 10% (w/v) stacking gels, comprising 4% (w/v) peptidoglycan. Samples were mixed 1:1 (v/v) with nonreducing sampling buffer [2.8 mL H_2O, 1 mL 0.5 mol/L Tris–HCl, pH 6.8, 3.2 mL sodium dodecyl sulfate (SDS; 10% w/v), 0.5 mL bromphenol blue (1%) and 7 mL glycerol] and were incubated at room temperature for 10 min. Gels were loaded with 10 µL of sample. Electrophoresis was performed using a Mini Protean Cell (Bio-Rad, Hempstead, UK) at a constant 60 V for approximately 2 h. Afterward, gels were rinsed briefly with distilled water and then washed twice in renaturation buffer (5% w/v Triton × in ddH_2O) for 20 min to remove the SDS and to allow the enzyme to renature again. Incubation of gels was carried out overnight in incubation buffer (phosphate buffer: 100 mmol/L; pH 7.5) at 37 °C. After incubation, gels were routinely stained with Coomassie blue (0.25% w/v) and destained in a solution containing 10% (v/v) acetic acid, 50% (v/v) dH_2O, and 40% (v/v) ethanol. Digestion of peptidoglycan was detected as a clearing zone in a dark blue background.

The procedure was optimized with commercial lysozyme (313–5000 U/mL), which was also used as a standard. After optimization, infected and noninfected wound fluid samples were measured. Additionally, the activity of commercial collagenase was investigated.

2.4. Labeling of M. lysodeikticus cells

2.4.1. Remazol brilliant blue

Labeling of bacterial cells was based on a standard all-in dyeing process. Briefly, a solution of 50 mg *M. lysodeikticus* cell wall was suspended in 0.5 mL RBB solution (0.5% w/v in ddH_2O). Sodium sulfate [Na_2SO_4, 2.5% (w/v)] and sodium carbonate [$NaCO_3$, 1% (w/v)] were dissolved in deionized water and directly added to the RBB-dyed peptidoglycan (PG-RBB) suspension with the equal volume. Dyeing was carried out in a thermoshaker (Eppendorf, Hamburg, Germany), using the following thermal gradient: 25 °C for 10 min and 65 °C for 5 min. The reaction mixture was centrifuged for 5 min at 11 357 × *g* to remove nonbonded dyestuff. The supernatant was discarded and the pellet was washed with deionized H_2O until the supernatant was colorless. The amount of dyestuff not being bound to peptidoglycan was determined to be 2.91 mg. The RBB content was therefore calculated to be 14% (w/w).

2.5. Preparation of agarose/peptidoglycan layers

2.5.1. Agarose/peptidoglycan-based monolayers

2.5.1.1. Unstained agarose/peptidoglycan-based monolayers.
Unstained agarose/peptidoglycan blend layers were prepared as follows. Agarose was used as matrix for the blends and was dissolved in phosphate buffer (100 mmol/L; pH 7.0) by heating in the microwave oven. Thereafter, different concentrations of peptidoglycan, ranging from 15 to 50 mg [0.15–0.50% (w/w)], were suspended in the 10 g of agarose solution (1% (w/w) and were mixed properly.

Preparation of the blends was directly carried out in 96-well plates. Therefore, different volumes of hot agarose–peptidoglycan suspension (60–150 µL) were directly transferred into the wells and were allowed to polymerize overnight. This protocol was optimized based on incubation temperature, layer thickness, and peptidoglycan concentration. Optimization was carried out regarding the increase of transparency of the matrix upon incubation with commercial lysozyme.

2.5.1.2. Stained agarose/peptidoglycan-based monolayers.
Stained agarose/peptidoglycan blend layers were prepared similarly by using stained peptidoglycan (PG-RBB) prepared as described above. For optimization, different stained peptidoglycan concentrations ranging from 2% to 40% (w/w), as well as different agarose

concentrations ranging from 0.25% to 1.00% (w/v), were used upon incubation with commercial lysozyme.

2.5.2. Agarose/peptidoglycan-based double layers

A double-layer system was prepared directly in microtiter plates composed of stained and unstained agarose/peptidoglycan. For this approach, 100 μL PG-RBB–agarose suspension [50 mg PG-RBB/2.5 g agarose, 2% (w/v)] was covered with 50 μL unstained PG–agarose suspension [50 mg PG-RBB/2.5 g agarose 2% (w/v)].

2.6. Stained alginate/peptidoglycan-based beads

Peptidoglycan-loaded alginate blend beads were prepared as follows. Briefly, an aqueous solution comprising 0.75% (w/v) alginate was prepared by stirring the solution at 25 °C for approximately 4 h. Before use, the solution was left to stand to remove disturbing trapped air bubbles. Thereafter, RBB-stained peptidoglycan [8% (w/w)] was added and the suspension mixed. Beads were prepared at room temperature by dropping a hot PG-RBB–alginate solution into 500 mL of an agitated calcium chloride solution [5% (w/v)], using a peristaltic pump equipped with a needle. The formed beads were left in the calcium solution for 30 min and were then rinsed with deionized water. Beads were stored till further use at 4 °C. The RBB content of the beads was calculated to be 20 ng RBB per gram of beads.

2.7. Enzymatic hydrolysis of stained and unstained agarose/peptidoglycan layers and alginate/peptidoglycan beads

To optimize composition and preparation conditions of agarose/peptidoglycan layers and alginate/peptidoglycan beads, in vitro degradation experiments were performed with commercial lysozyme from chicken white egg (AppliChem, Darmstadt, Germany) at 37 °C and shaking at 350 rpm. In addition, the influence of commercial neutrophil-elastase (Sigma, St. Louis, MO, USA), collagenase type IV (GIBCO™ Invitrogen), and MMP-9 (Sigma) was investigated.

2.7.1. Enzymatic hydrolysis of agarose/peptidoglycan monolayers and double layers

2.7.1.1. Unstained agarose/peptidoglycan-based monolayers. Different amounts of polymer solution were used per well and dried overnight in the fridge. Two hundred microliters of enzyme solution (lysozyme or wound fluid samples diluted 1:10 with buffer) were added to the polymer for incubation. Degradation of peptidoglycan due to lysozyme activity was monitored as increase in transparency, measured at 450 nm. Measurements took place directly in the reaction plate for 120 min.

2.7.1.2. Stained agarose/peptidoglycan-based monolayers. RBB-stained agarose/peptidoglycan layers casted in microtiter plates were incubated at 37 °C with 200 μL of lysozyme (ranging from 312 to 5000 U/mL) or wound fluid samples diluted 1:10 with buffer. Every 15 min, 100 μL of

sample was taken, transferred into a microtiter plate, and measured at 600 nm. All measurements were repeated 6-fold.

2.7.2. Enzymatic hydrolysis of alginate/peptidoglycan beads

The release of dye–PG fragments with different lysozyme activities was tested. Therefore, 9 beads (79.39 ± 8.34 mg) of RBB-stained beads were incubated at 37 °C with 200 μL lysozyme (5000, 2500, 1250, 625, 312, and 0 U/mL) in Eppendorf tubes at 350 rpm. In addition to lysozyme, the effect of collagenase, elastase, and MMPs on the hydrolysis of agarose/peptidoglycan beads was tested. Therefore, RRB-PG beads were incubated with lysozyme (250 U/mL) together with collagenase (10 U/mL), elastase (10 U/mL), and MMPs, respectively. The release of soluble blue fragments into the supernatant was measured at 600 nm at given time intervals. All measurements were repeated 6-fold. Additionally, similar experiments were performed with wound fluid samples diluted 1:10.

2.8. Long-term stability tests of alginate/peptidoglycan beads and agarose/peptidoglycan monolayers

The stability of RBB-stained alginate/peptidoglycan beads and agarose/peptidoglycan blend layers in microtiter plates that were stored at 4 °C was determined. Microtiter plates and beads that were aliquoted in Eppendorf tubes were stored at 4 °C. Every week, beads and layers were incubated with 200 μL of lysozyme (10 000 U/mL) at 37 °C. After 30 min of incubation, OD_{600} was measured.

2.9. Direct infusion mass spectrometry analysis

To investigate the influence of staining of M. lysodeikticus cells on lysozyme-catalyzed hydrolysis, reaction products were analyzed with a liquid chromatography–mass spectrometry (LC-MS) in direct injection mode. The MS spectra were acquired with an Agilent Ion Trap SL (Palo Alto, CA, USA) equipped with an electrospray ionization operated in positive and negative ion mode and the electrospray voltage was set to 3500 V. Dry gas flow was set to 9 L min^{-1} with a temperature of 350 °C, nebulizer set to 40 psi. Maximum accumulation time was fixed to 300 ms and the loading of the trap was controlled by the instrument with an ICC (ion charge control) of 30 000. Data were collected for 2.5 min/sample between 50 and 400 Da/e in a single scan with a scan rate of 0.02 min per scan. For analysis, the recorded mass data were averaged. Fifty milligrams stained and unstained PG was incubated in 1.5 mL phosphate buffer with lysozyme (6666 U/mL). Incubation was carried out at 37 °C for 12 h. The supernatants were transferred into glass vials and vials were placed in the autosampler of the LC system for automation of the MS injection.

2.10. Western blot

Western blot analysis was performed for detecting MMP-9 in wound fluid. Proteins were separated via SDS–polyacrylamide gel electrophoresis (PAGE) by molecular

weight. They were then transferred to a membrane and detected by protein–antibody interaction. The ECL™ Western blotting Analysis System was provided by Amersham Pharmacia Biotech (Uppsala, Sweden).

For SDS-PAGE, 7.5% SDS gels (0.75 mm) were used. Per sample, 5–50 μg protein was applied. Samples were mixed with 2.5 μL 10× sample buffer and were diluted with ddH$_2$O to a volume of 25 μL. Wound fluids as well as purified MMP-9 (gelatinase B, 92-kDa type IV collagenase; Chemicon Gelatinase kit, Temecula, CA, USA) and standards (broad range, low range) were heated at 95 °C for 10 min. Ten microliters of sample solution and 8 μL of standard solution were loaded, respectively. Electrophoresis was conducted at a voltage of 150 V (73 mA) with the Bio-Rad apparatus.

After separation, proteins on the gels were blotted onto the membrane in the blotting cell (BIO) with 350 mA for 1 h. Nonspecific binding sites were blocked in 0.5% nonfat dried milk overnight in the fridge. After washing, incubation with the primary antibody solution (Chemicon Gelatinase kit, diluted 1:5000) for 90 min was performed. After extensive washing of the membranes, they were incubated with peroxidase-conjugated goat antirabbit IgG (1:15000). Protein bands were detected using the ECL™ Western blotting Analysis System (Amersham Pharmacia Biotech) with subsequent exposure to X-ray film.

2.11. Statistical analysis

To compare the differences of lysozyme activity in infected and noninfected wound fluids, paired t test was performed. $P \leq 0.005$ was found to be statistically significant.

3. Results

3.1. Enzyme activity in wound fluids

Lysozyme activities of infected and noninfected wounds were determined according to Shugar (1952), while commercial lysozyme was used as standard in this procedure. Thirteen wounds out of 26 were labeled as noninfected wounds while 13 wounds were described as clinically infected by the attending doctors. As shown in (Table 1), lysozyme activity in infected wound fluid was significantly higher than in noninfected wounds ($P \leq 0.005$),

thus according well with the clinical description. There was no significant difference between blisters and noninfected postoperative or decubitus wounds regarding lysozyme levels. Similarly, there were no significant differences of lysozyme levels in infected wounds from decubitus or postoperative wounds.

To confirm the differences found in lysozyme levels, a zymogram-based assay was performed. We and others have successfully developed and used zymogram-based techniques involving renaturation of enzymes for a variety of other polysaccharide hydrolyzing enzymes (Pricelius et al., 2009). Fig. 1 shows a zymogram of a polyacrylamide gel containing [4% (w/v)] M. lysodeikticus cell wall with commercial lysozyme and wound fluid samples.

Lysozyme-catalyzed hydrolysis of peptidoglycan incorporated into SDS gels leads to observable clearing zones in a blue-stained background after staining. The intensity correlated well to the enzyme activity applied (Fig. 1) Additionally, this method was successfully used for wound fluid samples, thus allowing semiquantitative measurement of lysozyme activity. Only infected wound fluid samples showed clear digestion zones, while noninfected samples did not (data not shown). As a negative control, bovine serum albumin failed to yield clear spots indicating that the clearing spots are not artifacts of electrophoresis or refolding.

3.2. Diagnostic devices based on enzymatically controlled dye release

3.2.1. Device A: transparency increase of agarose/peptidoglycan blend layers

For the development of a simple diagnostic tool, the liquid lysozyme assay was adapted to as solid system. Therefore, in a first stage, the composition of agarose/peptidoglycan layers was optimized. An approach comprising 0.45% (w/v) peptidoglycan and casting in microtiter plates at 45 °C turned out most successful. A higher content of peptidoglycan led to inhomogeneous distribution of peptidoglycan, with PG particles on the surface. Additionally, no correlation between increase of transparency and lysozyme activity could be observed. On the

Table 1
Lysozyme activity in wound fluid samples

Clinical description of the wound	B	PO	D	L activity (U/mL)	L concentration (μg/mL)
Infected	0	8	5	4830 ± 1848	24.15 ± 9.24
Noninfected	8	2	2	376 ± 240	1.79 ± 1.22

Lysozyme (L) activity (U/mL) was measured in wound fluid samples of blisters (B), postoperative (PO), and decubitus (D) wounds. There is a significant difference between lysozyme activity of infected and noninfected wounds ($P \leq 0.005$).
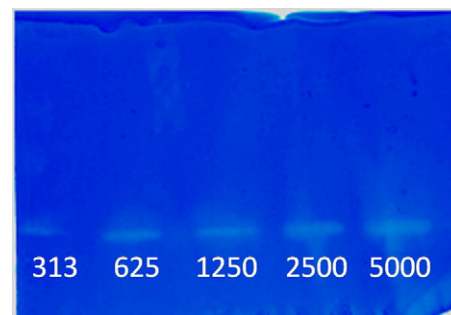

Fig. 1. (A) Zymogram of M. lysodeikticus: zymogram gels containing 4% (w/v) M. lysodeikticus cell wall were loaded with different lysozyme solutions, ranging from 313 to 5000 U/mL.
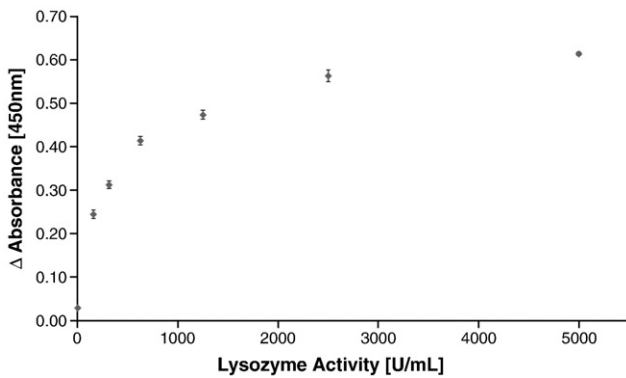
Fig. 2. Correlation of transparency increase and lysozyme activity: transparency increase of agarose/peptidoglycan blend layers [0.45% (w/w)] after incubation with various lysozyme activities at 37 °C for 2 h measured at 450 nm.
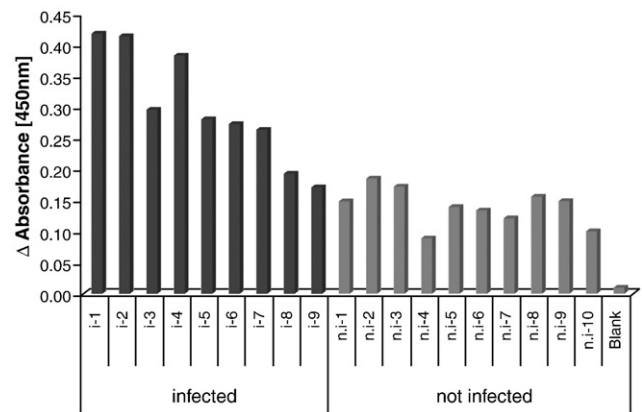

Fig. 3. Distinction of infected and noninfected wound fluid samples: transparency increase (450 nm) of agarose/peptidoglycan blend layers [0.45% (w/w)] after incubation with infected (i) and noninfected (n.i) wound fluid samples at 37 °C for 2 h. Average absorbance changes of infected wound fluid samples are 2-fold higher than those of noninfected wounds ($P \leq 0.005$).

other hand, a lower content resulted in layers with already too high transparency. A layer thickness of 2.0 mm was appropriate for visual inspection of a transparency change within 30 min of incubation. Using these conditions, the transparency changes depending on enzyme activity of commercial lysozyme (Fig. 2) and wound fluids (Fig. 3) were monitored.

3.2.2. Device B: dye release from agarose/peptidoglycan monolayers

To improve the detection sensitivity of the above system, agarose/peptidoglycan monolayers were constructed using peptidoglycan covalently dyed with RBB. It is known that RBB binds preferentially to 6-hydroxyl groups of hexoses (Hardt et al., 2003). In our study, the RBB content was calculated to be 14% (w/v). Therefore, every fourth disaccharide and every eighth saccharide of the peptidoglycan backbone was stained with one molecule of RBB. For both unstained PG and RBB-stained PG, the major hydrolysis product apart from higher oligomers was identified by LC-MS to be the disaccharide (m/z 496.9) consisting of N-acetylglucosamine and N-acetylmuramic acid (Fig. 4). This indicates that partial derivatization of peptidoglycan with RBB did not sterically affect hydrolysis by lysozyme.

Different amounts of stained PG [8–40% (w/v)] were tested for optimization of the device. The optimal concentration of stained PG turned out to be 8% (w/w) and was therefore used for all further experiments. Higher amounts of stained PG resulted in an unspecific release of dye even in the absence of enzyme. Incubation of this optimized system was carried out with different concentrations of commercial lysozyme. A linear correlation regarding the dye released up to 2500 U/mL was observed (Fig. 5). Using lysozyme activities higher than 2500 U/mL, the absorbance increase remained static, which could be due to steric hindrance or inaccessibility. Additionally, diluted (1:10) wound fluid samples of infected and noninfected wounds were investi-

gated regarding its ability for controlled release of the dye (Fig. 6).

3.2.3. Device C: dye release from agarose/peptidoglycan double layers

To avoid separation of the supernatant and spectrophotometric analysis of which, a system was constructed allowing simple visual judgment of wound infection. Therefore, peptidoglycan/agarose double layers were constructed. The lower layer contained stained PG, and the upper layer, unstained PG. Consequently, these layers appeared white to pale yellow from the top.

Upon incubation with high lysozyme levels, the upper layer was hydrolyzed, and subsequently, the blue peptidoglycan from the lower layer appeared, yielding to a color change from pale yellow to dark blue (Fig. 7). Similarly, incubation of infected wound samples resulted in the same change from white to dark blue after 120 min.

3.2.4. Device D: dye release from alginate/peptidoglycan beads

To reduce incubation time, beads comprising dyed peptidoglycan in alginate were constructed. Because of a higher surface area, beads should lead to a faster release of dye (or dyed fragments) upon incubation with lysozyme compared to the layers described above. Again, peptidoglycan 8% (w/w) was most suitable, while a higher PG content led to an inhomogeneous suspension resulting in difficulties in beads preparation.

The color release from stained alginate/peptidoglycan beads after incubation for 60 min and 120 min with different lysozyme activities and wound fluid samples is shown in Figs. 8 and 9.

Much higher absorbance changes were reached after incubation of beads for 2 h, compared to stained peptidoglycan layers, as expected (Fig. 8). Additionally, incubation for
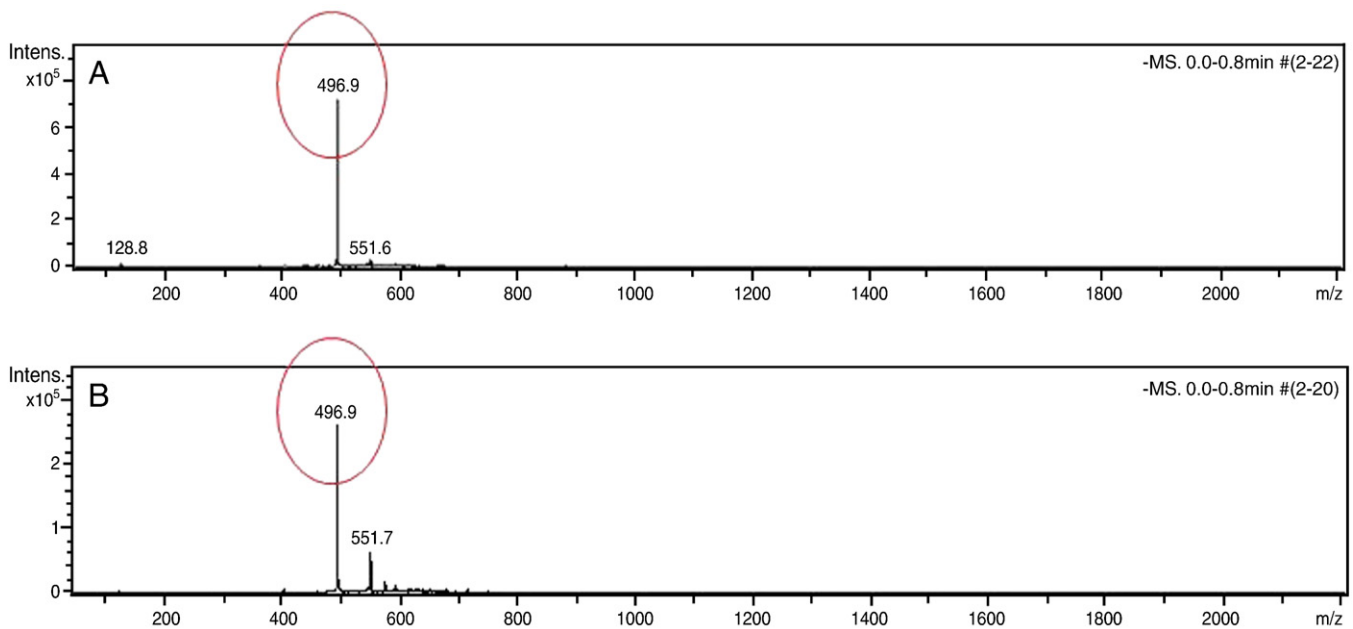
Fig. 4. MS-spectrum of digested RBB-stained (A) and unstained peptidoglycan (B): staining procedure of peptidoglycan did not influence the degradation ability of lysozyme (6666 U/mL).

60 min led to higher absorbance changes than incubation of device B for 120 min, which is probably due to a higher surface area. This behavior was observed for commercial lysozyme as well as for wound fluids. Additionally, a broad linear range of the calibration curve could be achieved. With proceeding hydrolysis the absorbance further increased while a maximum for all enzyme activities was reached after 24 h of incubation (data not shown). Concerning wound fluid samples, a clear difference between infected and noninfected wounds was reached (Fig. 9). The difference between infected and noninfected wound fluid samples was much more pronounced compared to stained peptidoglycan layers (factor 7 compared to factor 4). Apart from spectrophotometric quantification of released dye and dye–PG fragments,

this color change can be easily seen in the system. Therefore, beads could be used for the construction of, for example, test stripes, allowing diagnosis based on direct visual inspection. As stability is a crucial fact for application of the system, this behavior was tested. Beads were stable for at least 3 months and showed no loss of reactivity.

3.3. Influence of proteases on digestion of peptidoglycan

Wound fluids are a very complex matrix, comprising a great number of different biomolecules. Neutrophil-derived elastase and MMPs are the major proteases present in chronic wounds playing an important role in delayed wound
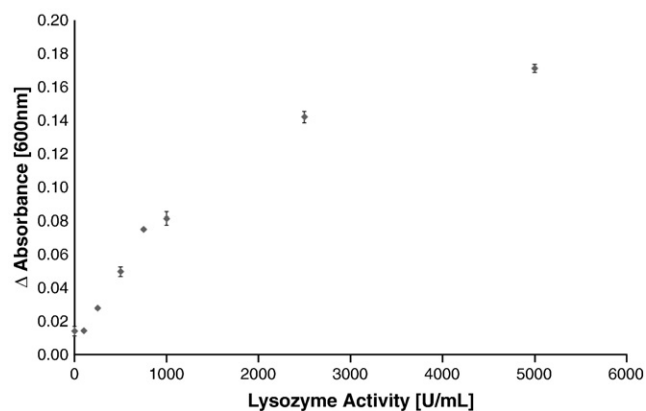

Fig. 5. Correlation of color release and lysozyme activity: color release from stained agarose/peptidoglycan blend layers [8% (w/w)] was measured at a wavelength of 600 nm after incubation with different lysozyme activities (313–10 000 U/mL) at 37 °C for 120 min.
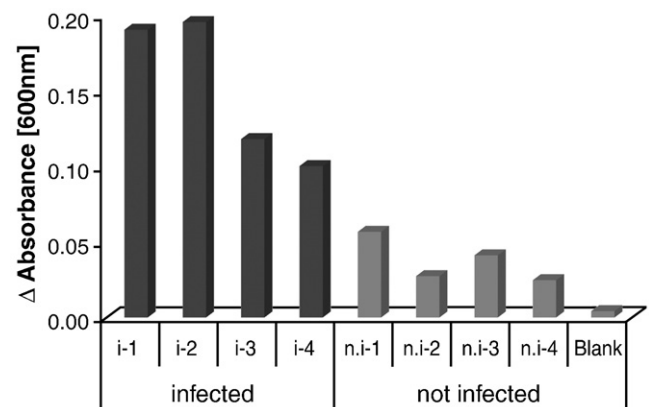

Fig. 6. Distinction of infected and noninfected wound fluid samples: color release from stained [8% (w/v)] agarose/peptidoglycan blend monolayers after incubation with infected (i-1 to i-5) and noninfected (n.i-1 to n.i-4) wound fluid samples for 2 h at 37 °C. Average absorbance changes of infected wound fluid samples are 4-fold higher than those of noninfected wounds ($P \leq 0.005$).
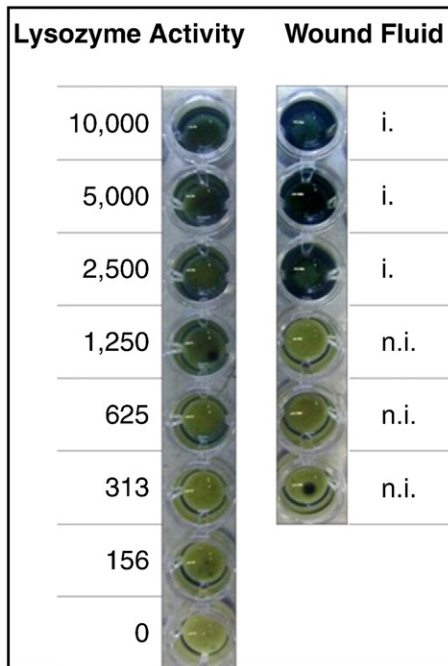
Fig. 7. Visual detection approach: layer alignment of stained and unstained peptidoglycan in microtiter plates. Double layers were incubated with different lysozyme activities (156–10 000 U/mL) and with 3 infected (i.) and 3 noninfected (n.i.) wound fluid samples.

healing. Elevated levels of different proteinases (elastase, cathepsin G, gelatinases, and collagenases) in infected and chronic wounds are well documented. While acute wounds show low levels of MMPs and elastase (Barrick et al., 1999; Trengove et al., 1999; Yager and Nwomeh,1999), chronic wounds contain excessive amounts of MMPs as shown by Wysocki et al. (1993) and Trengove et al. (1999). High levels of MMP activity in chronic wounds decrease as soon as wounds heal.

Because of the presence of peptide bonds in cell wall peptidoglycan, the influence of elastase and collagenase potentially present in wound fluid samples was investigated. Indeed, in infected wound samples, elastase activity of 1.82 U/mL and gelatinase activity of 10.5 U/mL were measured. As can be seen in Fig. 10, incubation of stained peptidoglycan beads with lysozyme and gelatinase MMP-9 as well as with elastase, respectively, showed an improved hydrolysis of remazol-dyed peptidoglycan.

3.4. Western blot

To determine the nature of gelatinase activity potentially contributing to hydrolysis of PG, Western blot analysis was carried out. Purified human gelatinase MMP-9 as a standard was detected between 66.2 and 97.4 kDa and was thus verified as the 92-kDa MMP-9 proenzyme. Additionally, MMP-9 was detected in infected wound fluid samples (Fig. 11).

4. Discussion

4.1. Enzyme activity in wound fluids

Lysozyme (EC 3.2.1.17) belongs to the enzyme class of gycosidases that are able to catalyze the hydrolysis of the glycosidic linkages between *N*-acetylmuramic acid and *N*-acetylglucosamine residues of the bacterial cell wall peptidoglycan (Salton, 1961). One of the biological functions of lysozyme is believed to be self-defense against infection by bacteria (Osserman et al., 1973), as it is normally released from monocytes, macrophages, and polymorphonuclear leukocytes (Haneberg et al., 1984; Torsteinsdottir et al., 1999). Elevated serum lysozyme levels are well documented for different diseases like tuberculosis and sarcoidosis (Osserman and Lawlor, 1966; Pascual et al., 1973). Therefore, the measurement of lysozyme concentration in serum or urine is useful for the diagnosis or screening
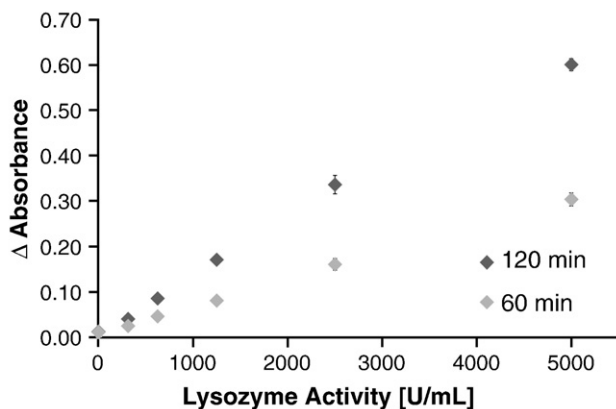

Fig. 8. Correlation of color release and lysozyme activity: color release from stained [8% (w/v)] agarose/peptidoglycan beads after incubation with different lysozyme activities ranging from 313 to 5000 U/mL at 37 °C for 60 and 120 min.
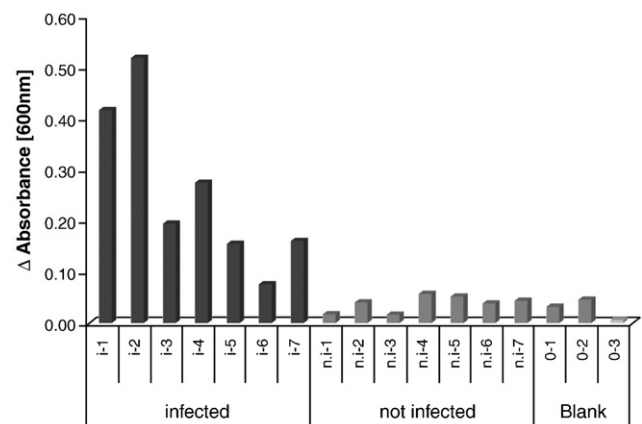

Fig. 9. Distinction of infected and noninfected wound fluid samples: color release from stained 8% (w/v) alginate/peptidoglycan beads after incubation with infected (i-1 to i-5) and noninfected (n.i-1 to n.i-4) wound fluid samples for 2 h at 37 °C. Average absorbance changes of infected wound fluid samples are 7-fold higher than those of noninfected wounds ($P \leq 0.005$).
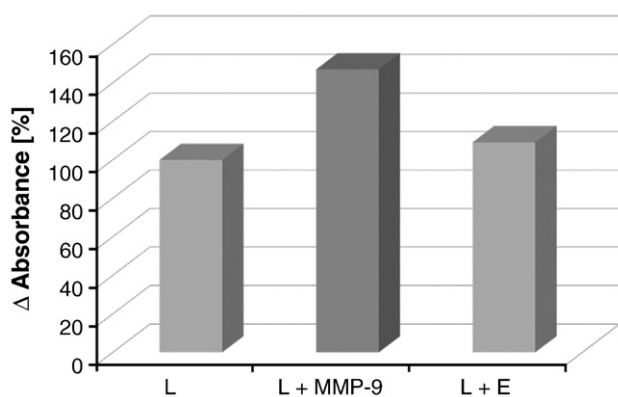
Fig. 10. Influence of different proteinases on release of dye fragments: alginate/peptidoglycan beads were incubated with MMP-9 and elastase (E) together with lysozyme (L) and compared with lysozyme L alone (250 U/mL) after incubation for 28 h at 37 °C.

of diseases. In addition, raised lysozyme levels were identified as a serum marker of active chronic inflammation (Selroos and Klockars,1977; Torsteinsdottir et al., 1999; Turton et al., 1979). Furthermore, sensitivity of lysozyme as a marker for sarcoidosis was shown to be high (Gronhagen-Riska and Selroos, 1979; Tomita et al., 1999).

Wound infection is one of the most common reasons for the nonhealing of a wound, leading to death in the worst case. It is widely assumed that wound fluid has the potential to provide information that can be used as a diagnostic indicator for the overall status of a wound or as prognostic indicators (Trengove et al., 2002; Yager et al., 2007). In detail, Trengove et al. (2002) found comparable levels of the C-reactive protein in serum and wound fluid. C-reactive protein is a widely used marker for inflammation. Additionally,

neutrophils and monocytes are predominant cell markers of wound infection (Hart, 2002; Sylvia, 2003). Here we found clearly different lysozyme levels in wound fluid of clinically infected and noninfected wounds. Thus, this observed difference between infected and noninfected wounds probably reflects the number of monocytes/macrophages and neutrophils in the tissue. The significantly elevated lysozyme levels in infected wounds show the potential of this parameter for diagnosis of wound infection and prompted us to use lysozyme for the development of different enzyme-responsive devices based on enzymatically controlled release for a simple detection of this enzyme.

4.2. Diagnostic devices based on enzymatically controlled dye release

4.2.1. Device A: transparency increase of agarose/peptidoglycan blend layers

Using unstained agarose/peptidoglycan blend layers, infected and noninfected wound fluid samples showed observable and significant ($P \leq 0.005$) differences in the change of absorbance after 2 h of incubation. There is a clear tendency of high absorbance changes in case of infected wound fluids. However, the absorbance changes obtained in 2 out of 9 infected samples are rather close to those of noninfected samples. The decrease of the shape and the short linear region (up to 300 U/mL) of the calibration curve of this system seemed to be the limiting factor as we found lysozyme levels around 4000 U/mL in infected wounds. Because blue-labeled *M. lysodeikticus* cells has previously shown higher sensitivity in turbidimetric assays or incorporated in zymograms (Hardt et al., 2003; Ito et al., 1992), staining of peptidoglycan was considered to improve the detection.

4.2.2. Device B: dye release from agarose/peptidoglycan monolayers

Using stained peptidoglycan, the linearity of the calibration curve could be increased compared to the unstained system. This improvement of the system is of great importance for a precise differentiation between infected and noninfected wounds. With this system, a clear difference ($P \leq 0.005$) between infected and noninfected wound fluids was detected regarding the release of dye. The difference with this system was indeed much more pronounced (factor 4) when compared to the system involving unstained PG (factor 2).

4.2.3. Device C: dye release from agarose/peptidoglycan double layers

Successfull incubation of device C was carried out using different lysozyme activities, yielding in a color change from yellow to dark blue (Fig. 7). Similarly, incubation of infected wound samples resulted in the same change from white to dark blue after 120 min.

As all devices described above use agarose as solid matrix, possible contribution of agarases to hydrolysis of the
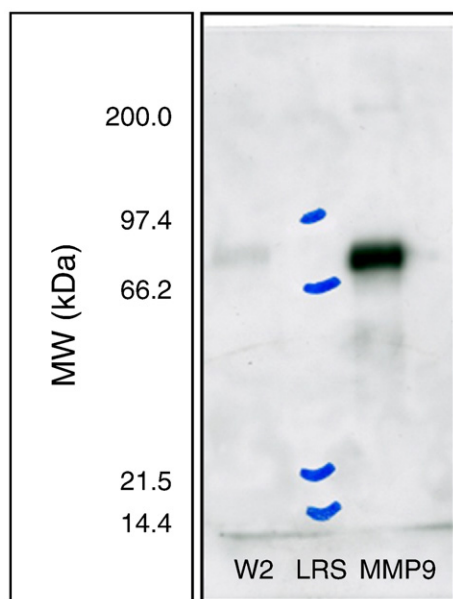

Fig. 11. Detection of MMP-9 via Western blot. W2, infected wound fluid sample; LRS, low range standard; MMP9, purified MMP-9 proenzyme.

matrix should be considered. Agarases are capable of degrading agarose and are found in bacteria like *Pseudomonas* sp., *Bacillus* sp., *Alteromonas*, and *Vibrio*. As *Pseudomonas* spp. are known to cause infection in chronic wounds (Goodman et al., 2004), their presence in wounds and secretion of agarases could enhance response of this system due to hydrolysis of agarose (Carlsson and Malmqvist, 1977; Hassairi et al., 2001; Malmqvist, 1978).

4.2.4. Device D: dye release from alginate/peptidoglycan beads

To reduce incubation time, beads comprising dyed peptidoglycan in alginate were constructed. Because of a higher surface area, beads should lead to a faster release of dye (or dyed fragments) upon incubation with lysozyme compared to the layers described above. Again, peptidoglycan 8% (w/v) was most suitable, while a higher PG content led to an inhomogeneous suspension resulting in difficulties in beads preparation.

Much higher absorbance changes were reached after incubation of beads for 2 h, compared to stained peptidoglycan layers, as expected. Additionally, incubation for 60 min led to higher absorbance changes than incubation of device B for 120 min, which is probably due to a higher surface area. This behavior was observed for commercial lysozyme as well as for wound fluids. Additionally, a broad linear range of the calibration curve could be achieved. With proceeding hydrolysis the absorbance further increased while a maximum for all enzyme activities was reached after 24 h of incubation. Concerning wound fluid samples, a clear difference between infected and noninfected wounds was reached. The difference between infected and noninfected wound fluid samples was much more pronounced compared to stained peptidoglycan layers (factor 7 compared to factor 4). Apart from spectrophotometric quantification of released dye and dye–PG fragments, this color change can be easily seen in the system. Therefore, beads could be used for the construction of, for example, test stripes, allowing diagnosis based on direct visual inspection. As stability is a crucial fact for application of the system, this behavior was tested. Beads were stable for at least 3 months and showed no loss of reactivity.

Again, the influence of alginolytic enzymes or so-called alginases has to be considered. The following bacteria are known for producing alingate lyase: *Pseudomonas* sp., *Bacillus* sp., *Sphingomonas* sp., *Streptomyces* sp., *Klebsiella* sp. (Cao et al., 2007; Sawabe et al., 1997; Wong et al., 2000). The presence of the above organisms in wounds could enhance the response of the system due to secretion of alginases partially degrading peptidoglycan/alginate beads.

4.3. Influence of proteases on digestion of peptidoglycan

Because of the presence of peptide bonds in cell wall peptidoglycan, the influence of elastase and collagenase potentially present in wound fluid samples was investigated.

Indeed, in infected wound samples, elastase activity of 1.82 U/mL and gelatinase activity of 10.5 U/mL were measured. Incubation of stained peptidoglycan beads with lysozyme and gelatinase MMP-9 as well as with elastase, respectively, showed an improved hydrolysis of remazol-dyed peptidoglycan.

Wound fluids are a very complex matrix, comprising a great number of different biomolecules. Neutrophil-derived elastase and MMPs are the major proteases present in chronic wounds playing an important role in delayed wound healing. Elevated levels of different proteinases (elastase, cathepsin-G, gelatinases, and collagenases) in infected and chronic wounds are well documented. While acute wounds show low levels of MMPs and elastase (Barrick et al., 1999; Trengove et al., 1999; Yager and Nwomeh,1999), chronic wounds contain excessive amounts of MMPs as shown by Wysocki et al. (1993) and Trengove et al. (1999). High levels of MMP activity in chronic wounds decrease as soon as wounds heal.

Interestingly, the addition of different proteases to lysozyme led to a synergistic increase in the release of dye and dye–PG fragments, while collagenase type IV showed no effect (data not shown). Especially the combination of MMP-9 and lysozyme enhanced the hydrolysis of peptidoglycan dramatically. In the presence of MMP-9 and lysozyme, the absorbance measured at 600 nm increased 40% compared to lysozyme alone. Additional incubation with neutrophil elastase led to an increase of 10%. A possible explanation for this observed effect is the ability of proteases to hydrolyze peptide bonds of PG as is described well in the literature for PG peptidases (Shockman and Höltje, 1994). PG hydrolases are enzymes with a broad variety of enzyme specificities, including peptidases as well as glycosidases (Ghuysen et al., 1966). Glycosidases are capable of cleaving glycosidic bonds between the sugar units of PG, whereas peptidases cleave peptide bonds of the pentapeptide.

4.4. Western blot

To determine the nature of gelatinase activity potentially contributing to hydrolysis of PG, Western blot analysis was carried out. Enhanced levels of MMP-9 has previously been reported in human blood originating from neutrophils upon addition of PG from *Staphylococcus* (Wang et al., 2005) as well as in chronic wound fluid (Wysocki et al., 1993). Purified human gelatinase MMP-9 as a standard was detected between 66.2 and 97.4 and was thus verified as the 92-kDa MMP-9 proenzyme. Additionally, MMP-9 was detected in infected wound fluid samples. Thus, a contribution of MMP-9 to hydrolysis of PG is possible.

5. Conclusion

Significantly elevated levels of lysozyme, elastase, and gelatinase were detected in infected wound fluids. The sensitivity and specificity of lysozyme detection in wound fluid samples were enhanced by using RBB-labeled

peptidoglycan/agarose layers or peptidoglycan/alginate beads. Using a double-layer system, wound infection can be indicated by simple visual inspection. A synergistic effect in dye release has been found for MMP-9 and neutrophil elastase, which would enhance the response of the system in wound fluid. Such a diagnostic tool would allow early intervention with suitable treatment and could reduce clinical intervention and the use of antibiotics. The tool is therefore based on human enzymes, which can be detected in wound fluid samples in a very fast way and which were shown to be elevated in case of infection.

Acknowledgment

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